Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	11	glnap$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:38
L2	286	acetyl adj phosphate or acetylphosphate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L3	114394	promoter$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L4	1815924	induc$8 or regulat$8 or activat$8 or modulat$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L5	2	2 same 3 same 4	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L6	418982	acetate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37
L9	12633	isoprenoid$1 or lycopene$1 or carotene$1 or astaxanthin$1 or phytoene$1 or isopentyl adj diphosphate or ipp	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:43
L10	43684	polyhydroxybutyrate$1 or phb$1 or polyhydroxyvalerate$1 or phv$1 or polyhydroxyalkanoate$1 or pha$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L11	1246	polyketide$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L12	4	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 2	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:13
L13	159	(9 or 10 or 11) near4 (gene$1 or sequence$1) and (6 same 4)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:11
L14	45	(9 or 10 or 11) near4 (gene$1 or sequence$1) same (2 or 6)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:14
L15	931	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 6	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:11
L16	51	15 and carbon adj (flux or flow)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:15

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	11	glnap$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:38

PGPUB-DOCUMENT-NUMBER: 20050106560

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20050106560 A1

TITLE: Selection methods

PUBLICATION-DATE: May 19, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wohlstadter, Jacob Nathaniel	Andover	MA	US	

APPL-NO: 10/ 876343

DATE FILED: June 23, 2004

RELATED-US-APPL-DATA:

child 10876343 A1 20040623

parent continuation-of 09573830 20000518 US GRANTED

parent-patent 6846628 US

child 09573830 20000518 US

parent continuation-of 08235437 19940429 US GRANTED

parent-patent 6087177 US

US-CL-CURRENT: 435/5, 435/252.3 , 435/472

ABSTRACT:

A rational method for obtaining a novel molecule capable of a desired interaction with a substrate of interest comprising selecting hosts or replicators which encode said novel molecules based upon cell or replicator growth caused by the desired interaction of the novel molecule and a selection molecule expressed by said host.

---------- KWIC ---------

Detail Description Paragraph - DETX (300):

[0367] The cells are then incubated in glutamine and nitrogen limiting media. Cells capable of growth are selected. In this example selection occurs through a cascade. A properly phosphorylated NR.sub.I is capable of acting as an enhancer to the promoter glnApZ recognized by .sigma..sup.54 by binding an upstream DNA sequence. The enhanced glnApZ promoter then allows for the high level expression of glnA (glutamine synthetase) and DNaA. These proteins then in turn activate cascades which allow for cellular growth and replication.

PGPUB-DOCUMENT-NUMBER: 20050032217

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20050032217 A1

TITLE: Generation of human regulatory T cells by bacterial toxins for the treatment of inflammatory disorders

PUBLICATION-DATE: February 10, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Zadeh, Homayoun H.	Calabasas	CA	US	

APPL-NO: 10/ 817506

DATE FILED: April 1, 2004

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60459778 20030401 US

US-CL-CURRENT: 435/455, 435/252.3 , 435/372 , 435/471

ABSTRACT:

An adoptive immunotherapy using ex vivo-generated regulatory T cells may be used for the suppression of undesireable immune response. T cells are to be obtained from the patient's blood, and upon exposure to a set of toxins from the pathogen A. actinomycetemcomitans, the population of regulatory T cells will be enriched ex vivo and adoptively transferred back to the patient. The novel aspect of the present invention is that it generates large numbers of type 1 regulatory T cells, which secrete Interleukin-10.

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Ser. No. 60/459,778, filed Apr. 1, 2003, the content of which is incorporated herein by reference.

---------- KWIC ---------

Detail Description Paragraph - DETX (71):

[0103] In an alternate embodiment, live vaccines prepared in accordance with the preferred embodiment which have non-reverting mutations in genes under nitrogen control may be used as vectors or carriers for antigens of species other than the non-virulent pathogenic vaccine. One or more structural genes coding for the desired antigens may be operatively linked to an ntr-regulated promoter. As the non-virulent pathogenic microbe senses a nitrogen deficiency, expression of the ntr genes increases accordingly, thus initiating expression of genes transcribed from ntr promoters. For example, one or more structural genes coding for the desired antigens may be operatively linked to the glnAp2 promoter located on a suicide vector or an autonomously replicating plasmid. The suicide vector or plasmid may then be introduced into the non-virulent pathogenic strain having non-reverting mutations by well known methods, such as, transduction, transformation, electroporation, tri-parental mating

techniques or direct transfer of a self mobilized vector in a bi-parental mating and maintained within the microorganism either autonomously or in the bacterial host's chromosomal DNA.

US-PAT-NO: 6846628

DOCUMENT-IDENTIFIER: US 6846628 B1

TITLE: Selection methods

DATE-ISSUED: January 25, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Wohlstadter; Jacob Nathaniel	Andover	MA	01810	N/A

APPL-NO: 09/ 573830

DATE FILED: May 18, 2000

PARENT-CASE:

This application is a continuation division of application Ser. No. 08/235,437, filed Apr. 29, 1994, now U.S. Pat. No. 6,087,177.

US-CL-CURRENT: 435/6, 435/320.1

ABSTRACT:

A rational method for obtaining a novel molecule capable of a desired interaction with a substrate of interest comprising selecting hosts or replicators which encode said novel molecules based upon cell or replicator growth caused by the desired interaction of the novel molecule and a selection molecule expressed by said host.

9 Claims, 9 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 9

---------- KWIC ---------

Detailed Description Text - DETX (292):

The cells are then incubated in glutamine and nitrogen limiting media. Cells capable of growth are selected. In this example selection occurs through a cascade. A properly phosphorylated NR.sub.I is capable of acting as an enhancer to the promoter glnApZ recognized by .sigma..sup.54 by binding an upstream DNA sequence. The enhanced glnApZ promoter then allows for the high level expression of glnA (glutamine synthetase) and DNaA. These proteins turn activate cascades which allow for cellular growth and replication.

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L2	286	acetyl adj phosphate or acetylphosphate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L3	114394	promoter$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L4	1815924	induc$8 or regulat$8 or activat$8 or modulat$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L5	2	2 same 3 same 4	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L6	418982	acetate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37

PGPUB-DOCUMENT-NUMBER: 20050084884

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20050084884 A1

TITLE: MEKK1 molecules and uses thereof

PUBLICATION-DATE: April 21, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Palombella, Vito J.	Needham	MA	US	
Liao, Sha-Mei	Lexington	MA	US	

APPL-NO: 10/ 930194

DATE FILED: August 31, 2004

RELATED-US-APPL-DATA:

child 10930194 A1 20040831

parent continuation-of 09697898 20001027 US GRANTED

parent-patent 6818427 US

US-CL-CURRENT: 435/6, 435/194 , 435/320.1 , 435/325 , 435/69.1 , 536/23.2

ABSTRACT:

The invention provides full-length, human isolated nucleic acids molecules, designated MEKK1 nucleic acid molecules, which encode a MEKK family member. The invention also provides antisense nucleic acid molecules, recombinant expression vectors containing MEKK1 nucleic acid molecules, host cells into which the expression vectors have been introduced, and nonhuman transgenic animals in which a MEKK1 gene has been introduced or disrupted. The invention still further provides isolated MEKK1 proteins, fusion proteins, antigenic peptides and anti-MEKK1 antibodies. Diagnostic methods utilizing compositions of the invention are also provided.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX (9):

[0007] MEKK1 activates both the Activator Protein-1 (AP-1) stress response pathway and the NF.kappa.B pathway. The transcription factor AP-1 is a critical regulator of T-cell activation, cytokine production, including IL-2, IL-3, and GM-CSF, and the production of metalloproteinases. (Gottschalf et al., J. Exp. Med. 178:1681 (1993); Want et al., J. Mol. Cell. Biol. 14:11153 (1994); Rao, Immunol. Today 15:274 (1994); Angel et al., Biochem. Biophys. Acta. 1072:129 (1991).) With regard to cytokine regulation, AP-1 mediates positive transactivation independently or in association with NF-AT (Nolan, Cell 77:795 (1994)). AP-1 activity is induced by many stimuli, including the phorbol ester tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA), growth factors, cytokines, T-cell activators, neurotransmitters, and UV irradiation. AP-1 is composed of dimers of different members of the Fos and Jun family of proteins. AP-1 activity is regulated at the level of both C-Jun

and C-Fos transcription and by post-translational modification of their protein products by phosphorylation and dephosphorylation.

PGPUB-DOCUMENT-NUMBER: 20040253608

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20040253608 A1

TITLE: Compounds with anti-KS and anti-HIV activity

PUBLICATION-DATE: December 16, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Antakly, Tony	Montreal		CA	
Sairam, Ram M.	Dollard-des-Ormeaux		CA	

APPL-NO: 10/ 733323

DATE FILED: December 12, 2003

RELATED-US-APPL-DATA:

child 10733323 A1 20031212

parent division-of 09494500 20000131 US GRANTED

parent-patent 6683050 US

child 09494500 20000131 US

parent continuation-of PCT/CA98/00731 19980730 US UNKNOWN

non-provisional-of-provisional 60054543 19970801 US

US-CL-CURRENT: 435/6, 424/185.1 , 424/187.1 , 435/5 , 514/2 , 530/326 , 530/350

ABSTRACT:

The present invention relates to a compound having anti-KS and anti-HIV pharmaceutical activity which comprises an HCG-like inhibitory protein and fragments or derivatives thereof, said protein and fragments thereof are isolated from a biologically active fraction of APL-HCG, wherein said protein has a molecular weight of about 3,500 or of about 13,000 Dalton, and wherein said protein and fragments thereof are adsorbed polypropylene plastic supports. A pharmaceutical composition for the prevention and/or treatment of Kaposi's sarcoma (KS) and HIV which comprises an therapeutically effective amount of at least one compound of the present invention in association with a pharmaceutically acceptable carrier. A method for the prevention, treatment and/or reduction of Kaposi's sarcoma and HIV expression in AIDS patients, which consists in administering the composition to the patient.

[0001] This application is a continuation of PCT/CA98/00731 filed Jul. 30, 1998 designating the United States and claiming priority of U.S. provisional Patent Application Ser. No. 60/054,543 filed Aug. 1, 1997.

---------- KWIC ---------

Detail Description Paragraph - DETX (30):

[0074] Activating protein-1 (AP-1) is a transcriptional activator which is induced by 12-O-tetradecanyl phorbol-13-acetate (TPA) tumor promoter, several growth factors and various extracellular stimuli (reviewed in Saatcioglu F et al., 1994, Semin. Cancer Biol. 5:347-359). AP-1 consists of proteins of jun and fos families which associate to form homo-(jun/jun) or heterodimers (jun/fos) and recognize a consensus sequence 5'-TGA G/C TCA-3' known as TPA Response Element (TRE) present on AP-1 regulated genes. AP-1 complexes are considered to play important roles in several signal transduction pathways such as growth stimulation, differentiation, neuronal excitation and transformation (Saatcioglu F et al., 1994, Semin. Cancer Biol. 5:347-359). APL-HCG and components in fraction 7 significantly inhibited AP-1 binding to TRE in KSY-1 cells (FIG. 2D). APL-HCG inhibited AP-1 binding by 1.5, 3 and 2 fold respectively after 3, 6 and 12 hours of treatment (FIG. 3).

US-PAT-NO: 6818427

DOCUMENT-IDENTIFIER: US 6818427 B1

TITLE: MEKK1 molecules and uses thereof

DATE-ISSUED: November 16, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Palombella; Vito J.	Needham	MA	N/A	N/A
Liao; Sha-Mei	Lexington	MA	N/A	N/A

APPL-NO: 09/ 697898

DATE FILED: October 27, 2000

US-CL-CURRENT: 435/194, 435/252.3 , 435/320.1 , 435/325 , 536/23.2

ABSTRACT:

The invention provides full-length, human isolated nucleic acids molecules, designated MEKK1 nucleic acid molecules, which encode a MEKK family member. The invention also provides antisense nucleic acid molecules, recombinant expression vectors containing MEKK1 nucleic acid molecules, host cells into which the expression vectors have been introduced, and nonhuman transgenic animals in which a MEKK1 gene has been introduced or disrupted. The invention still further provides isolated MEKK1 proteins, fusion proteins, antigenic peptides and anti-MEKK1 antibodies. Diagnostic methods utilizing compositions of the invention are also provided.

20 Claims, 5 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 8

---------- KWIC ---------

Brief Summary Text - BSTX (9):

MEKK1 activates both the Activator Protein-1 (AP-1) stress response pathway and the NF.kappa.B pathway. The transcription factor AP-1 is a critical regulator of T-cell activation, cytokine production, including IL-2, IL-3, and GM-CSF, and the production of metalloproteinases. (Gottschalf et al., J. Exp. Med. 178:1681 (1993); Want et al., J. Mol. Cell. Biol. 14:11153 (1994); Rao, Immunol. Today 15:274 (1994); Angel et al., Biochem. Biophys. Acta. 1072:129 (1991).) With regard to cytokine regulation, AP-1 mediates positive transactivation independently or in association with NF-AT (Nolan, Cell 77:795 (1994)). AP-1 activity is induced by many stimuli, including the phorbol ester tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA), growth factors, cytokines, T-cell activators, neurotransmitters, and UV irradiation. AP-1 is composed of dimers of different members of the Fos and Jun family of proteins. AP-1 activity is regulated at the level of both C-Jun and C-Fos transcription and by post-translational modification of their protein products by phosphorylation and dephosphorylation.

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	11	glnap$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:38
L2	286	acetyl adj phosphate or acetylphosphate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L3	114394	promoter$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L4	1815924	induc$8 or regulat$8 or activat$8 or modulat$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L5	2	2 same 3 same 4	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L6	418982	acetate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37
L9	12633	isoprenoid$1 or lycopene$1 or carotene$1 or astaxanthin$1 or phytoene$1 or isopentyl adj diphosphate or ipp	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:43
L10	43684	polyhydroxybutyrate$1 or phb$1 or polyhydroxyvalerate$1 or phv$1 or polyhydroxyalkanoate$1 or pha$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L11	1246	polyketide$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L12	4	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 2	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:54

PGPUB-DOCUMENT-NUMBER: 20040077090

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20040077090 A1

TITLE: Whole cell engineering by mutagenizing a substantial portion of a starting genome, combining mutations, and optionally repeating

PUBLICATION-DATE: April 22, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Short, Jay M.	Rancho Santa Fe	CA	US	

APPL-NO: 10/ 383798

DATE FILED: March 6, 2003

RELATED-US-APPL-DATA:

child 10383798 A1 20030306

parent continuation-of 09677584 20000930 US ABANDONED

child 09677584 20000930 US

parent continuation-in-part-of 09594459 20000614 US GRANTED

parent-patent 6605449 US

child 09594459 20000614 US

parent continuation-in-part-of 09522289 20000309 US GRANTED

parent-patent 6358709 US

child 09522289 20000309 US

parent continuation-in-part-of 09498557 20000204 US PENDING

child 09498557 20000204 US

parent continuation-in-part-of 09495052 20000131 US GRANTED

parent-patent 6479258 US

non-provisional-of-provisional 60156815 19990929 US

US-CL-CURRENT: 435/471, 435/252.3 , 435/254.2

ABSTRACT:

An invention comprising cellular transformation, directed evolution, and screening methods for creating novel transgenic organisms having desirable properties. Thus in one aspect, this invention relates to a method of generating a transgenic organism, such as a microbe or a plant, having a

plurality of traits that are differentially activatable. Also, a method of retooling genes and gene pathways by the introduction of regulatory sequences, such as promoters, that are operable in an intended host, thus conferring operability to a novel gene pathway when it is introduced into an intended host. For example a novel man-made gene pathway, generated based on microbially-derived progenitor templates, that is operable in a plant cell. Furthermore, a method of generating novel host organisms having increased expression of desirable traits, recombinant genes, and gene products.

---------- KWIC --------

Detail Description Paragraph - DETX (158):

[0327] Due to the severe limitations and problems related to having PAGE as an integral and central part in the standard DNA sequencing protocol, several methods have been proposed to do DNA sequencing without an electrophoretic step. One approach calls for hybridization or fragmentation sequencing (Bains, Biotechnology 10, 757-58 (1992) and Mirzabekov et al., FEBS Letters 256, 118-122 (1989)) utilizing the specific hybridization of known short oligonucleotides (e.g., octadeoxynucleotides which gives 65,536 different sequences) to a complementary DNA sequence. Positive hybridization reveals a short stretch of the unknown sequence. Repeating this process by performing hybridizations with all possible octadeoxynucleotides should theoretically determine the sequence. In a completely different approach, rapid sequencing of DNA is done by unilaterally degrading one single, immobilized DNA fragment by an exonuclease in a moving flow stream and detecting the cleaved nucleotides by their specific fluorescent tag via laser excitation (Jett et al., J. Biomolecular Structure & D3mamics 7, 301-309, (1989), United States Department of Energy, PCT Application No. WO 89/03432). In another system proposed by Hyman Anal. Biochem. 174, 423-436 (1988)), the pyrophosphate generated when the correct nucleotide is attached to the growing chain on a primer-template system is used to determine the DNA sequence. The enzymes used and the DNA are held in place by solid phases (DEAE-Sepharose and Sepharose) either by ionic interactions or by covalent attachment. In a continuous flow-through system, the amount of pyrophosphate is determined via bioluminescence (luciferase). A synthesis approach to DNA sequencing is also used by Tsien et al. (PCT Application No. WO 91/06678). Here, the incoming dNTP's are protected at the T-end by various blocking groups such as acetyl or phosphate groups and are removed before the next elongation step, which makes this process very slow compared to standard sequencing methods.

Detail Description Paragraph - DETX (954):

[1121] In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in

length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and in vitro studies of these genes/proteins.

Detail Description Paragraph - DETX (955):
[1122] The ability to select and combine desired components from a library of polyketides, or fragments thereof, and postpolyketide biosynthesis genes for generation of novel polyketides for study is appealing. The method of the present invention makes it possible to facilitate the production of novel polyketide synthases through intermolecular recombination.

Detail Description Paragraph - DETX (956):
[1123] Preferably, gene cluster DNA can be isolated-from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of E. coli. This f-factor of E. coli is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

Detail Description Paragraph - DETX (1821):
[1988] In other embodiments, indicator cells can in turn produce something that modifies the growth rate of the library cells via a feedback mechanism. Growth rate feedback can detect and accumulate very small differences. For example, if the library and reporter cells are competing for nutrients, library cells producing compounds to inhibit the growth of the reporter cells will have more available nutrients, and thus will have more opportunity for growth. This is a useful screen for antibiotics or a library of polyketide synthesis gene clusters where each of the library cells is expressing and exporting a different polyketide gene product.

Detail Description Paragraph - DETX (2824):
[2988] In other embodiments, indicator cells can in turn produce something that modifies the growth rate of the library cells via a feedback mechanism. Growth rate feedback can detect and accumulate very small differences. For-example, if the library and reporter cells are competing for nutrients, library cells producing compounds to inhibit the growth of the reporter cells will have more available nutrients, and thus will have more opportunity for growth. This is a usefull screen for antibiotics or a library of polyketide synthesis gene clusters where each of the library cells is expressing and exporting a different polyketide gene product.

Detail Description Paragraph - DETX (2848):
[3012] Gene clusters such as those involved in polyketide synthesis (or indeed any multi-enzyme pathways catalyzing is analogous metabolic reactions) can be recombined by recursive sequence recombination even if they lack DNA sequence homology. Homology can be introduced using synthetic oligonucleotides as PCR primers. In addition to the specific sequences for the gene being

amplified, all of the primers used to amplify one type of enzyme (for example the acyl carrier protein in polyketide synthesis) are synthesized to contain an additional sequence of 20-40 bases 51 to the gene (sequence A) and a different 20-40 base sequence 31 to the gene (sequence B). The adjacent gene (in this case the keto-synthase) is amplified using a 51 primer which contains the complementary strand of sequence B (sequence B'), and a 31 primer containing a different 20-40 base sequence (C). Similarly, primers for the next adjacent gene (keto-reductases) contain sequences C' (complementary to C) and D. If 5 different polyketide gene clusters are being stochastic &/or non-stochastic mutagenized, all five acyl carrier proteins are flanked by sequences A and B following their PCR amplification. In this way, small regions of homology are introduced, making the gene clusters into site specific recombination cassettes. Subsequent to the initial amplification of individual genes, the amplified genes can then be mixed and subjected to primeness PCR. Sequence B at the 3' end of all of the five acyl carrier protein genes can anneal with and prime DNA synthesis from sequence BI at the 5' end of all five keto reductase genes. In this way all possible combinations of genes within the cluster can be obtained. Oligonucleotides allow such recombinants to be obtained in the absence of sufficient sequence homology for recursive sequence recombination described above. Only homology of function is required to produce functional gene clusters.

Detail Description Paragraph - DETX (2854):

[3018] The cassette-based recombination method can be combined with recursive sequence recombination by including gene fragments (generated by DNase, physical shearing, DNA stuttering, etc.) for one or more of the genes. Thus, in addition to different combinations of entire genes within a cluster (e.g., for polyketide synthesis), individual genes can be stochastic &/or non-stochastic mutagenized at the same time (e.g., all acyl carrier protein genes can also be provided as fragmented DNA), allowing a more thorough search of sequence space.

Detail Description Paragraph - DETX (3397):

[3561] Isoprenoids result from cyclization of farnesyl pyrophosphate by sesquiterpene synthases. The diversity of isoprenoids is generated not by the backbone, but by control of cyclization. Cloned examples of isoprenoid synthesis genes include trichodiene synthase from Fusarium sprorotrichioides, pentalene synthase from Streptomyces, aristolochene synthase from Penicillium roquefortii, and epi-aristolochene synthase from N. tabacum (Cane, D. E. (1995). Isoprenoid antibiotics, pages 633-655, in "Genetics and Biochemistry of Antibiotic Production" edited by Vining, L. C. & Stuttard, C., published by Butterworth-Heinemann). Recursive sequence recombination of sesquiterpene synthases will be of use both in allowing expression of these enzymes in heterologous hosts (such as plants and industrial microbial strains) and in alteration of enzymes to change the cyclized product made. A large number of isoprenoids are active as antiviral, antibacterial, antifungal, herbicidal, insecticidal or cytostatic agents. Antibacterial and antiftngal isoprenoids could thus be preferably screened for using the indicator cell type system described above, with the producing cell competing with bacteria or fungi for nutrients. Antiviral isoprenoids could be screened for preferably by their ability to confer resistance to viral attack on the producing cell.

Detail Description Paragraph - DETX (3400):

[3564] Like polyketide synthases, peptide synthases are modular and multifunctional enzymes catalyzing condensation reactions between activated building blocks (in this case amino acids) followed by modifications of those building blocks (see Kleinkauf, H. and von Dohren, H. Eur. J. Biochem. 236:335-351 (1996)). Thus, as for polyketide synthases, recursive sequence recombination can also be used to alter peptide synthases: modifying the

specificity of the amino acid recogrized by each binding site on the enzyme and altering the activity or substrate specificities of sites that modify these amino acids to produce novel compounds with antibiotic activity other peptide antibiotics are made ribosomally and then post-translationally modified. Examples of this type of antibiotics are lantibiotics (produced by gram positive bacteria such Staphylococcus, Streptomyces, Bacillus, and Actinoplanes) and microcins (produced by Enterobacteriaceae). Modifications of the original peptide include (in lantibiotics) dehydration of serine and threonine, condensation of dehydroamino acids with cysteine, or simple N- and C-terminal blocking (microcins). For ribosomally made antibiotics both the peptide-encoding sequence and the modifying enzymes may have their expression levels modified by recursive sequence recombination. Again, this will lead to both increased levels of antibiotic synthesis, and by modulation of the levels of the modifying enzymes (and the sequence of the ribosomally synthesized peptide itself) novel antibiotics.

Detail Description Paragraph - DETX (3404):

[3568] Examples of starting materials for recursive sequence recombination include but are not limited to genes from bacteria such as Alcaligenes, Zoogloea, Rhizobium, Bacillus, and Azobacter, which produce polyhydroxyalkanoates (PHAs) such as polyhyroxybutyrate (PHB) intracellularly as energy reserve materials in response to stress. Genes from Alcaligenes eutrophus that encode enzymes catalyzing the conversion of acetoacetyl CoA to PIIB have been transferred both to E. coli and to the plant Arabidopsis thaliana (Poirier et al. Science 256:520-523 (1992)). Two of these genes (phbB and phbC, encoding acetoacetyl-CoA reductase and PHB synthase respectively) allow production of PHE in Arabidopsis. The plants producing the plastic are stunted, probably because of adverse interactions between the new metabolic pathway and the plants' original metabolism (i.e., depletion of substrate from the mevalonate pathway). Improved production of PHB in plants has been attempted by localization of the pathway enzymes to organelles such as plastids. Other strategies such as regulation of tissue specificity, expression timing and cellular localization have been suggested to solve the deleterious effects of PHB expression in plants. The recursive sequence recombination techniques of the invention can be used to modify such heterologous genes as well as specific cloned interacting pathways (e.g., mevalonate), and to optimize PHB synthesis in industrial microbial strains, for example to remove the requirement for stresses (such as nitrogen limitation) in growth conditions.

Detail Description Paragraph - DETX (3557):

[3721] In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides. Polyketides are molecules which are an extremely rich source of bioactivities, including antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase

genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and in vitro studies of these genes/proteins.

Detail Description Paragraph - DETX (3558):

[3722] The ability to select and combine desired components from a library of polyketides, or fragments thereof, and postpolyketide biosynthesis genes for generation of novel polyketides for study is appealing. The method of the present invention makes it possible to facilitate the production of novel polyketide synthases through intermolecular recombination.

Detail Description Paragraph - DETX (3560):

[3724] Preferably, gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of E. coli: This f-factor of E. coli is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

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ABSTRACT:

Disclosed are a variety of methods and computer systems for use in the analysis of gene and protein expression data. Also disclosed are methods for the definition of the cellular state of cells and tissues from multidimensional physiological data such as those obtained from gene expression measurements with DNA microarrays. A variety of classification methods can be applied to expression data to achieve this goal. Demonstrated is the application of several statistical tools including Wilks' lambda ratio of within-group to total variance, Fisher Discriminant Analysis, and the misclassification error rate to the identification of discriminating genes and the overall classification of expression data. Examples from several different cases demonstrate the ability of the method to produce well-separated groups in the projection space representing distinct physiological states. The method can be augmented and is useful in disease diagnosis, drug screening and bioprocessing applications.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. section 119(e) to Provisional Patent Applications 60/285,186 filed Apr. 20, 2001, and 60/264,779 filed Jan. 29, 2001. These applications are hereby incorporated by reference in their entirety.

---------- KWIC --------

Summary of Invention Paragraph - BSTX (15):

[0016] Polyhydroxyalkanoic acids (PHA) form a class of biopolymers, of which polyhydroxybutyric acid (PHB) is a member, that can be synthesized by many genera of bacteria and whose properties can vary over a large range. Doi, Y. 1990. Microbial Polyesters. VCH Publishers. Over 90 different members of the PHA class of biopolymers have been discovered each of which differs slightly in the number of carbons in the monomeric sub-unit or the structure of the pendant side chain. Steinbuchel, A., and B. Fuchtenbushc. 1998. Bacterial and other biological systems for polyester production. TIBTECH 16:419-426. PHAs are characterized by a polyester backbone and a diverse set of side-chain structures that provide considerable flexibility in PHA polymeric properties. Steinbuchel, A., and H. Valentin. 1995. Diversity of bacterial polyhydryoxyalkanoic acids. FEMS Microbiology Letters 128:219-228. Importantly, members of the PHA biopolymer family are biodegradable and are therefore of interest as an alternative to petrochemical based polymers. Biologically based processes for the production of PHAs have been established in the past 25 years with current bioproduction systems capable of PHA accumulation levels close to 80% of dry cell weight and productivities of almost 5 g/L-hr. Fidler, S., and D. Dennis. 1992. Polyhydroxyalkanoate production in recombinant Escherichia coli. FEMS Microbiology Reviews 9:231-235; Peoples, O., and A. Sinskey. 1989. Poly-beta-hydroxybutyrate biosynthesis in Alcaligenes eutrophus H16. Identification and characterization of the PHB polymerase gene (phbC). Journal of Biological Chemistry 264:15298-15393; Slater, S., T. Gallaher, and D. Dennis. 1992. Production of poly-(3-hydroxybutyrate-co-3-hydroxyva- lerate) in a recombinant Escherichia coli strain. Applied Environmental Microbiology 58:1089-1094; Stal, L. 1992. Poly(hydroxyalkanoate) in cyanobacteria: an overview. FEMS Microbiology Reviews 103:169-180; Steinbuchel, A., and B. Fuchtenbushc. 1998. Bacterial and other biological systems for polyester production. TIBTECH 16:419-426; Lee, S., J. Choi, and H. Wong. 1999. Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. International Journal of Biological Macromolecules 25:31-36; Liu, S., and A. Steinbuchel. 2000. A novel genetically engineered pathway for synthesis of poly(hydroxyalkanoic acids) in Escherichia coli. Appplied Environmental Microbiology 66:739-743; Park, S., W. Ahn, P. Green, and S. Lee. 2001. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyh- exanoate) by metabolically engineered Escherichia coli strains. Biotechnology and Bioengineering 74:81-86. One of the main limitations to the commercialization of PHA bioprocesses, however, has been -the use of expensive carbon substrates. Choi, J., and S. Lee. 2000. Economic considerations in the production of poly(3-hydroxybutyrate-co-3-hydroxyva- lerate) by bacterial fermentation. Applied Microbiology and Biotechnology 53:646-649; Lee, S., J. Choi, and H. Wong. 1999. Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. International Journal of Biological Macromolecules 25:31-36.

Summary of Invention Paragraph - BSTX (35):

[0035] In another aspect, the invention relates to a set of genes and a pattern of gene expression that is associated with differing levels of polyhydroxyalkanoate (PHA) production. Examples of such genes are provided in Table 1 below. In certain embodiments, the invention relates to altering the expression of one or more of these genes to improve PHA production, and in some embodiment, the altering involves the genetic makeup of the cell so as to cause the cell to have a modified expression of one of the genes. It is also understood that similar improvements in PHA production may be achieved through the manipulation of an orthologue of any of the preceding genes in the appropriate organism. A cell may be essentially any cell capable of producing

PHAs, optionally a cyanobacterial cell and optionally a bacterial cell of the species Synechocystis sp., Synechococcus sp., Ralstonia eutropha, Alcaligenes latus, Azotobacter vinelandii, Anacystis nidulans or recombinant Escherichia coli. PHAs might include, but are not limited to any of the following: polyhydroxyproprionate, polyhydroxybutyrate, polyhydroxyvalerate, polyhydroxycaproate, polyhydroxyheptanoate, polyhydroxyoctanoate, polyhydroxynonanoate, polyhydroxydecanoate, polyhydroxyundecanoate, polyhydroxydodecanoate and a mixed polymer of one or more of the forgoing polymers, and, for example 3-hydroxyproprionate, 3-hydroxybutyrate, 4-hydroxybutyrate, 5-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxycaproate, 3-hydroxyheptanoate, 3-hydroxyoctanoate, 3-hydroxynonanoate, 3-hydroxydecanoate, 3-hydroxyundecanoate and 3-hydroxydodecanoate.

Brief Description of Drawings Paragraph - DRTX (17):

[0055] FIG. 16: depicts transcript accumulation levels for **PHB biosynthetic genes** as described in reaction (A). PHB accumulation levels for the same conditions. PhaEC transcript accumulation level very closely followed PHB acumulation levels. Both phaAB and phaEC are bicistronic and good agreement between there values was observed.

Detail Description Paragraph - DETX (50):

[0104] As presented below, the analytical methods described herein are applicable to problems as diverse as the diagnosis of hyperproliferative cells, the classification of neoplasms, and the production of useful compounds by bacteria. Accordingly, in certain aspects, the invention provides genes, genes groups and patterns that are related to hyperproliferative states of epithelial cells (See Table 1), the distinction between forms of leukemias (See Table 2) In addition to identifying disease states such as oral cancer, use of projections such as those defined by Fisher Discriminant Analysis (FDA) in defining cellular physiological states from gene or protein expression measurements can also be used to systematically probe environmental conditions and the effects these conditions have on the cell's physiology. As described in the examples below, full genome DNA micro-arrays may now be used to profile transcriptional alterations in Synechocystis cells that have accumulated different levels of the biopolymer polyhydroxybutyrate (PHB) under varying nutritional conditions will help to develop metabolic engineering approaches to improve PHB accumulation. In certain embodiments, the invention provides genes, gene groups and patterns of **gene expression that are related to the production of polyhydroxyalkanoates** (See Table 3).

Detail Description Paragraph - DETX (186):

[0232] In certain aspects, the invention provides one or more genes that are related to a particular cellular state or change in cellular state. For example, the invention provides genes of Table 1 that are related to a hyperproliferative state in epithelial cells, genes of Table 2 that are related to different classes of leukemias and **genes of Table 3 that are related to polyhydroxyalkanoate** production. In some embodiments the invention also provides discriminatory patterns comprising one or more of the above genes or proteins encoded therein. In certain embodiments a different sample may be compared to the genes and patterns described herein, for the purpose of, for example, classifying the sample or evaluating a manipulation of the sample. Such comparisons may employ the variability-based statistics described above, or comparisons may be performed using any of the various statistical methods that, in view of this specification, may be selected by one of skill in the art.

Detail Description Paragraph - DETX (314):

[0350] Identification of **Genes Discriminatory of PHB** Accumulation Levels.

Detail Description Paragraph - DETX (326):
[0362] Changes in PHB-Related Genes.

Detail Description Paragraph - DETX (327):
[0363] PHB is synthesized in Synechocystis by the combined activities of four gene products from two bi-cistronic mRNA transcripts (FIG. 16). The phaAB (s1r1993 and s1r1994) genes code for the PHA specific b-ketothiolase and acetoacetyl-CoA reductase involved in the first steps of the PHA biosynthetic pathway. Taroncher-Oldenburg, G., K. Nishihara, and G. Stephanopoulos. 2001. Identification of a PHA specific b-ketothiolase and acetoacetylCoA reductase, phaEC, in Synechocystis sp. PCC6803. Applied Environmental Microbiology. The phaEC gene products comprise the PHA synthase which catalyzes the polymerization of hydroxybutyryl-CoA to form PHB. Hein, S., H. Tran, and A. Steinbuchel. 1998. Synechocystis sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Archives Microbiology 170:162-170. Transcripts from these genes accumulated preferentially in the conditions of maximum PHB accumulation, 10PA and 10P. This suggests at least some level of control at the transcriptional level for accumulation of the PHB biopolymer. Interestingly, trends within each bicistron (phaAB or phaEC) were remarkably consistent.

Detail Description Paragraph - DETX (332):
[0368] A closer examination of the genes in Table 3 demonstrates three categories of genes that are apparent: those such as cheY, encoding a chemotaxis protein expressed as an adaptation to nutrient limitation, but clearly not promising as a target gene for enhancing PHB synthesis in future studies; genes such as the transcriptional regulator hypF, likely to be involved in global cellular responses to nutrient starvation and a potential good candidate for improving PHB synthesis; and unannotated genes such as s110008 for which a function has not been ascribed. The last two categories of genes are potential targets for future metabolic engineering strategies for improving PHB production, which would not have been considered from a mechanistic approach concentrating solely on the enzymatic steps of the PHB synthesis pathway. This demonstrates the value of transcriptional profiling and FDA in target gene identification for metabolic engineering purposes.

Detail Description Paragraph - DETX (339):
[0375] The discovery of genes that collectively correlate with cell states of interest and, therefore, define targets for genetic control is an important focus of metabolic engineering. In this study we have reported the results of a number of different analytical approaches to determining such genes based on whole-genome transcriptional profiles. Specifically, we described results for discriminatory genes as determined by Fisher discriminatory analysis, phosphate related genes, nitrogen related genes, PHB biosynthesis genes, and for those genes which altered most dramatically in each of the conditions studied.

Detail Description Paragraph - DETX (340):
[0376] The conditions examined in this study were designed to allow for transcriptional profiling of cell states moderately limited in the different nutrients (starting at N or P sufficient conditions (10% (v/v)) and grown to early stationary phase) that are associated with PHB accumulation. Miyake, M., K. Kataoka, M. Shirai, and Y. Asada. 1997. Control of poly-beta-hydroxybutyrate synthase by acetyl phosphate in cyanobacteria. Journal of Bacteriology 179:5009-5013. At more dramatic starvation conditions, cell growth rate is reduced and final cell density is decreased to a level in which mRNA quality was not suitable for profiling by micro-arrays. Collier, J. Grossman, A. A small polypeptide triggers complete degradation of light-harvesting phycobiliproteins in nutrient-deprived cyanobacteria. EMBO J

13:1039-1047. Therefore, while our studies did force PHB accumulation by nutrient limitation, starvation conditions and the starvation response were not specifically examined. Moreover, in all cultures except 0.3%N we did not observe any chlorosis at the time of mRNA purification. Importantly, the lack of severe starvation conditions combined with the presence of differential PHB accumulation allowed for a clearer analysis of genes involved with biopolymer accumulation rather than cell starvation.

Detail Description Paragraph - DETX (341):

[0377] Overall, the use of FDA was shown to provide a concise list of genes which clearly discriminated for particular growth conditions. Similar results were not obtained when evaluated phosphate or nitrogen related genes. In fact, nitrogen related genes did not appear to reflect growth conditions in the majority of cases. The examination of genes which accumulated most dramatically in each of the conditions studied did not provide any useful information with regard to discrimination between PHB accumulation states. In contrast, PHB related genes did vary closely with PHB accumulation suggesting some level of transcriptional control.

Detail Description Paragraph - DETX (344):

[0380] Similar metabolic engineering strategies can be envisioned based on the results for phosphate related genes. In particular, we know that the phosphate limitation is reflected at the transcriptional level in only one of the phosphate transport systems while the second transport system was not substantially altered in the conditions studied. This second transport system, therefore, is potentially available for genetic manipulations aimed at improving growth in phosphate limited cultures by increasing phosphate transport. An additional target for genetic manipulation was the pta gene. This gene is known to be involved in activation of the PHB synthase enzyme and its transcripts were observed to accumulate in correlation with PHB accumulation conditions. Therefore, altering the level of this gene through overexpression or other means is proposed to have an affect of PHB accumulation.

Detail Description Paragraph - DETX (346):

[0382] The results for phaAB and phaEC in nitrogen limited conditions also presented an opportunity for further study and possible metabolic engineering. Specifically, in nitrogen limited cultures PHB biopolymer accumulated to close to 2% (DCW) even though transcript levels were not significantly altered from that of full BG11 media in which PHB accumulated to only 0.4% (DCW). Therefore, genetic manipulations aimed at increasing the levels of the PHB biosynthetic genes in Nitrogen limited growth conditions (among others) present a worthwhile opportunity for improving biopolymer accumulation.

Detail Description Paragraph - DETX (351):

[0386] One of the primary limitations in any metabolic engineering study is the selection of target genes for manipulation. One consistent outcome of such studies is the complexity of cell regulatory responses to our attempts to engineer metabolism. Transcriptional profiling has gained considerable attention as a means for target discovery in metabolic engineering among others (i.e. drug discovery). What has been unclear is the extent to which changes in transcript accumulation represent effects of physiological alterations as opposed to causing physiological alterations. In the absence of massive numbers of samples, this information can not be reasonably obtained in transcriptional profiling studies. What can be obtained, however, is a set of target genes which appear to 1) be important to the condition under study and 2) show substantial regulation at the transcriptional level. An additional criteria of importance is determining which genes are be coordinately overxpressed as a regulon to ensure proper ratios of their products in

engineered cells. Finally, protein expression studies are required to fully characterize the extent to which the gene-products are also differentially regulated. The genes described in this study provided a reduced set of targets when compared to the whole genome but a more detailed set when compared to strictly looking at the PHB biosynthetic pathway alone. This was demonstrated by comparing the results from the FDA to the results for the phosphate-related, nitrogen-related, and PHB synthesis genes. The FDA genes were all clearly discriminatory for specific nutrient conditions. Also, most of these genes were of no clear relation to the biosynthesis of PHB. As a result, a new target gene set was obtained which satisfied the criteria listed above and which could not have reasonably been obtained otherwise. The phosphate-related and PHB-related genes also showed some promise in terms of target selection even though any nitrogen-related gene targets were not obvious. We can reasonably conclude that studies such as these should rely upon data-driven approaches, such as FDA, for target discovery but can also benefit from an analysis of genes known to be involved in the pathways of interest.

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DOCUMENT-IDENTIFIER: US 6706516 B1

TITLE: Engineering of metabolic control

DATE-ISSUED: March 16, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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APPL-NO: 09/ 626612

DATE FILED: July 27, 2000

PARENT-CASE:

CROSS REFERENCE

This application claims priority from U.S. provisional application serial No. 60/145,801, filed Jul. 27, 1999.

US-CL-CURRENT: 435/252.33, 435/166 , 435/167 , 435/252.3 , 435/320.1 , 536/24.1

ABSTRACT:

The invention features a method of producing heterologous molecules in cells under the regulatory control of a metabolite and metabolic flux. The method can enhance the synthesis of heterologous polypeptides and metabolites.

5 Claims, 0 Drawing figures

Exemplary Claim Number: 1

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Brief Summary Text - BSTX (4):

The invention is based, in part, on the discovery that production of heterologous polypeptides and metabolites can be enhanced by the regulated expression of the polypeptide (e.g., a biosynthetic enzyme) using a promoter which is regulated by the concentrations of a second metabolite, e.g. acetyl phosphate. The term "heterologous" refers to a polypeptide or metabolite which is introduced by artifice. A heterologous polypeptide or metabolite can be identical to endogenous entity that is naturally present. The term "metabolite" refers to a organic compound which is the product of one or more biochemical reactions A metabolite may itself be a precursor for other reactions. A secondary metabolite is a metabolite derived from another.

Brief Summary Text - BSTX (6):

The host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. For example, the host cell can genetically modified by deletion or mutation of a gene encoding a histidine protein kinase, e.g., a member of COG0642 as defined by (http://www.ncbi.nlm.nih.gov/COG/; Tatusov et al. supra.), e.g., glnL, phoR,

phoQ, creC, or envZ. In another example, the histidine protein kinase has specificity for the response regulator protein which controls the promoter. The histidine protein kinase can be encoded by glnL, e.g., E coli glnL (SWISSPROT P06712; http://www.expasy.ch/).

Brief Summary Text - BSTX (7):
Whereas the host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation, for heterologous polypeptide or metabolite expression, the host cell can be propagated in any desired condition, e.g., in nitrogen starvation conditions, nitrogen poor conditions, or nitrogen rich conditions.

Brief Summary Text - BSTX (9):
Another aspect of the invention features a kit containing a nucleic acid sequence which includes a promoter controlled by a response regulator protein. The kit further optionally contains a bacterial host cell which is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. The kit can also provide instructions for their use. The nucleic acid sequence can contain a restriction enzyme polylinker located 3' of the promoter such that a sequence inserted into the polylinker is operably linked to the promoter which is controlled by a response regulator protein. In one implementation of the kit, the promoter is the E. coli glnAp.sub.2 promoter and the bacterial host cell is an E. coli cell containing a mutation or deletion of the glnL gene.

Brief Summary Text - BSTX (10):
Another aspect of the invention features a host cell containing a first expression cassette. The first expression cassette includes a promoter, such as any of those described above, and a nucleic acid sequence encoding an enzyme required for biosynthesis of a heterologous metabolite. As used herein, "enzyme" refers to a polypeptide having ability to catalyze a chemical reaction or multiple reactions. The nucleic acid sequence is operably linked to the promoter which is regulated by acetyl phosphate in the absence of nitrogen starvation. The host cell also contains additional nucleic acid sequences for expressing other enzymes required for biosynthesis of the metabolite. Such additional sequences may be endogenous sequences expressing endogenous enzymes, or introduced sequences expressing heterologous enzymes.

Brief Summary Text - BSTX (12):
The host cell can be a bacterial cell, e.g., an E. coli cell. The host cell is optionally genetically modified by deletion or mutation of a gene, e.g., a gene encoding a histidine protein kinase, as described above. In one specific example, the host cell further contains a second expression cassette containing a nucleic acid sequence encoding phosphoenolpyruvate synthase operably linked to a promoter regulated by acetyl phosphate concentration, e.g., glnAp.sub.2.

Brief Summary Text - BSTX (15):
In one implementation, the first metabolite is a polyhydroxyalkanoate, e.g., polyhydroxybutyrate and the nucleic acid sequence encodes a biosynthetic enzyme, e.g., a 3-ketoacyl coenzyme A (coA) reductases, or a poly-3-hydroxyoctanoyl-CoA polymerase. In another case, the first metabolite is a polyketide, a .beta.-lactamn antibiotic, or an aromatic. In a yet another case, the first metabolite is an isoprenoid, e.g., an isoprenoid mentioned herein. The nucleic acid sequence can encode a biosynthetic enzyme required for isoprenoid production, e.g., isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, 1-deoxyxylulose 5-phosphate synthase, phosphoenolpyruvate synthase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, or lycopene cyclase. One precursor of isoprenoids can be pyruvate. Pyruvate

concentrations are related to acetate and acetyl-phosphate concentrations. Accordingly, in this instance, the second metabolite is acetyl phosphate. The promoter responding to acetyl phosphate can be controlled by a response regulator protein, e.g., a response regulator protein mentioned above. Such a promoter may only respond to acetyl phosphate in a specific host cell. In a particular example, the promoter responding to acetyl phosphate concentration is bound by E. coli ntrC, e.g., E. coli glnAp.sub.2 promoter.

Brief Summary Text - BSTX (23):

If the expression cassette is to be used for engineering regulated production of a heterologous polypeptide during late logarithmic growth or during stationary phase, then the promoter can be chosen accordingly. For example, a promoter can be chosen that responds to small molecule signal, e.g., a second messenger, whose levels accumulate during late logarithmic growth or during stationary phase. The second messenger can be a molecule that accumulates as a precursor, an intermediate, or a waste product of a biochemical pathway. In bacteria, the small molecule signal can be a glycolysis intermediate, e.g., fructose 1-phosphate or fructose 6phosphate or a glycolysis waste product, e.g., acetate or acetyl phosphate. In eukaryotic cells, cAMP concentrations are a well known signal of nutrient state.

Brief Summary Text - BSTX (24):

The promoter in the expression cassette can be chosen based on the results of a large scale expression analysis experiment, e.g., a gene chip experiment. Genes which are induced by acetyl phosphate can be identified by hybridizing to a microarray labeled cDNA prepared from cells in grown in acetate and comparing the signal to a reference signal, e.g., to the signal of obtained with cDNA prepared from cells in early logarithmic growth. This experiment can be performed on both prokaryotic and eukaryotic cells, e.g., bacterial, yeast, plant and mammalian cells. For an example of such an experiment in a prokaryote, see Talaat et al. (2000) Nat Biotechnol 18:679-82 and Oh & Liao (2000) Biotechnol Prog. 16:278-86. Once a gene is identified which is expressed under the desired condition, its promoter can utilized in the expression cassette. Alternatively, the experiment can be performed by the exogenous addition of a desired molecule (e.g., a precursor in a metabolic pathway) or by manipulation of experimental conditions (e.g., growth to late logarithmic phase or growth while a biosynthetic enzyme is overproduced). Promoters can be identified based on the genes induced.

Brief Summary Text - BSTX (25):

In one instance, an expression cassette is used for engineering regulated production of a metabolite in a bacterial cell. The promoter can be selected which is regulated by a second metabolite whose concentration is indicative of the availability of a precursor for the biosynthesis of the first metabolite. For example, if the first metabolite is an isoprenoid which is synthesized from the precursors, pyruvate and glyceraldhyde 3-phosphate, then the second metabolite can be acetyl phosphate. In a rich environment, cells produce an excess amount of acetyl-CoA, a product of pyruvate. The excess acetyl-CoA is used to produce ATP and acetate, which is secreted as a waste product. Acetate concentration increases with cell density. Acetate, acetyl-CoA, and acetyl-phosphate concentrations are interrelated by to the following biochemical reactions: (1) acetyl-CoA+P.sub.i ⇄acetyl phosphate+CoA (2) acetyl phosphate+ADP⇄acetate+ATP

Brief Summary Text - BSTX (26):

Thus, high acetyl phosphate concentration is indicative of excess acetyl-CoA and excess pyruvate. A host cell which is genetically modified by deletion or mutation of glnL, for example, causes ntrC function to become acetyl phosphate dependent (Feng et al. (1992) J Bacteriol 174:6061-6070). In this fashion, a

promoter regulated by ntrC, e.g., the glnAp2 promoter, can be used to control gene expression in response to acetyl phosphate. The glnAp2 promoter can be obtained using standard techniques in the art. For example, primers can be designed and synthesized that anneal to the glnAp2 promoter. The polymerase chain reaction (PCR) can be used to amplify a nucleic acid fragment containing the glnAp2 promoter. This fragment can now be used for further constructions. Likewise, an E. coli strain containing deletion of histidine protein kinase gene, e.g., glnL can be easily prepared. See Link et al. (1997) J Bacteriol. 179(20):6228-6237 for a detailed description of one possible method. The sequences encoding a desired heterologous polypeptide can be cloned downstream of the glnAp2 promoter so that it is operably linked to the promoter. A host cell with an inactivated glnL gene can then be transformed with the sequences. The transformed strain can be grown, and polypeptide production monitored during the course of growth. Robust protein expression can be observed at high cell densities, as in Farrner and Liao (2000) Nat. Biolechnol 18:533-537, the contents of which are hereby incorporated by reference.

Detailed Description Text - DETX (8):
Increasing levels of acetyl phosphate can be an indicator of excess glucose flux. The current invention features host cells, nucleic acids sequences, and methods of utilizing acetyl phosphate as a signal to regulate the expression of rate-controlling enzymes in a desired metabolic pathway, both to utilize fully the excess carbon flux and to redirect the flux away from the toxic product, acetate.

Claims Text - CLTX (1):
1. A E. coli host cell comprising (i) a genetic alteration inactivating the glnL gene; (ii) a nucleic acid sequence comprising a coding sequence encoding a phosphoenol pyruvate synthetase (pps) and an operably linked glnAp2 promoter that is regulated by ntrC and acetyl phosphate; and (iii) nucleic acid sequences encoding a geranylgeranyl diphosphate synthase, a phytoene synthase, and a phytoene desaturase.

Other Reference Publication - OREF (6):
McCleary et al., "Acetyl phosphate and the activation of two-component response regulators," Journal of Biological Chemistry, vol. 269, No. 50, pp. 31567-31572 (1994).

Other Reference Publication - OREF (11):
McCleary et al., "Acetyl phosphate a global signal in Escherichia coli?," Journal of Bacteriology, vol. 175, No. 10, pp. 2793-2798 (1993).

Other Reference Publication - OREF (13):
Shin et al., "Modulation of Flagellar expression in Escherichia coli by acetyl phosphate and the osmoregulator OmpR," Journal of Bacteriology, vol. 177, No. 16, pp. 4696-4702 (1995).

Other Reference Publication - OREF (25):
Wanner et al., "Involvement of Phosphotransacetylase, Acetate Kinase, and Acetyl Phosphate Synthesis in Control of the Phosphate Regulon in Escherichia coli", J. Bacteriol. 174(7):2124-2130 (1992).

US-PAT-NO: 5891686

DOCUMENT-IDENTIFIER: US 5891686 A

TITLE: Method of production of poly-.beta.-hydroxyalkanoate copolymers

DATE-ISSUED: April 6, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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APPL-NO: 08/ 881562

DATE FILED: June 24, 1997

PARENT-CASE:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 08/610,804, filed Mar. 7, 1996, which is a continuation of U.S. patent application Ser. No. 08/042,236, filed Mar. 31, 1993 and now abandoned, which is a continuation of U.S. patent application Ser. No. 08/035,433, filed Mar. 24, 1993 and issued on Oct. 29, 1996 as U.S. Pat. No. 5,569,595 .

US-CL-CURRENT: 435/135, 435/252.3 , 435/252.34 , 435/252.35

ABSTRACT:

The present invention provides methods for the production of poly-.beta.-hydroxyalkanoate copolymer comprising the steps of (a) introducing into a prokaryotic host cell a vector construct which directs the expression of a sequence which encodes a poly-.beta.-hydroxybutyrate biosynthetic pathway, (b) introducing into the host cell a vector construct which directs the expression of one or more proteins which regulate acetate and propionate metabolism, (c) culturing the host cell in medium containing propionate or a derivative thereof, and (d) isolating poly-.beta.-hydroxyalkanoate copolymer from the cultured host cell.

14 Claims, 33 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 29

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Drawing Description Text - DRTX (2):
FIG. 1 is a chemical reaction sequence showing the synthesis of PHB.

Detailed Description Text - DETX (16):
As noted above, two other genes which encode proteins which regulate

acetate/propionate metabolism are ack and pta. Briefly, conversion of acetate to acetyl-CoA may be accomplished by two different enzymatic systems. The first is the acetate kinase/phosphotransacetylase system found in E. coli. In this system the acetate kinase (ack) converts acetate to acetyl phosphate, which is then converted to acetyl-CoA by the action of phosphotransacetylase (pta). The ack gene may be readily obtained as described below in the Examples. The pta gene may be readily obtained following the methods described by Yamamoto-Otake et al. in Applied Microbiology and Biotechnology 33:680-682, 1990. The second method for conversion of acetate to acetyl-CoA is via an acetyl-CoA synthetase. Normally, acetyl-CoA synthetase enzymes are inducible (by their substrate, acetate), and have significant activity using propionate as the substrate (Jetten et al., Journal of Bacteriology 171:5430-5435; 1989). As shown in the Examples below, the ack gene and pta gene may be utilized in order to obtain high levels of 3-HV incorporation into a copolymer.

Detailed Description Text - DETX (43):
PHB was grown in E. coli harboring the PHB biosynthetic genes under conducive conditions, i.e., a flask of LB is inoculated with the E. coli harboring the PHB biosynthetic pathway and the E. coli are grown in the presence of 1% glucose (where glucose acts as the carbon source for PHB production).

Detailed Description Text - DETX (47):
The p4A plasmid exists in the cell ate an abnormally high copy number (50-200 per cell) thereby increasing the gene dosage of the PHB biosynthetic genes resulting in extremely high PHB production (as high as 95% of the cell weight). Thus, p4A is a "multicopy plasmid." The term "multicopy plasmid" is used in the sense of the ordinary definition and means a plasmid which exists in a plural number in a host cell.

Detailed Description Text - DETX (49):
The plasmid p4A is superior to other plasmids based on its copy number, because in alkaline minipreps the plasmid yield from p4A is about twice as much as other PHB-plasmids. Gene dosage effect may be responsible for high levels of PHB production in E. coli. To test this hypothesis, the PHB pathway was cloned into plasmid pOU71 obtained from Dr. Soren Molin (Larsen et al., Gene 28:45, 1984). This plasmid is maintained as a single copy when grown at 30.degree. C. In experiments where the plasmid was maintained as a single copy, PHB production was 1/40th of that found in 04A in E. coli DH5alpha If the PHB genes are found in single copies in A. eutrophus, this indicates that the genes are not well-expressed in E. coli, but overcome this deficit by having a large number of genes. This is supported by reports that, in general, A. eutrophus genes are not well-expressed in E. coli, but overcome this deficit by having a large number of genes. This is supported by reports that, in general, A. eutrophus genes are poorly expressed in E. coli (Anderson et al., J. Bact. 159:97, 1984).

Detailed Description Text - DETX (52):
The enzymes needed for PHB-co-V production are not normally to be found in most host strains, including E. coli. However, acetyl-CoA synthetase is an inducible enzyme of the acetate utilization system in many host strains, including for example, Escherichia and Salmonella. According to the method of the present invention PHA production in the host is accomplished by inducing acetate utilization genes in a host and thereafter producing the PHAs by allowing the host cells containing the acetate utilization genes grow.

Detailed Description Text - DETX (112):
The amount of acetyl-hydroxamate made (a measure of acetate kinase activity) was calculated by adding hydroxylamine solution to known amounts of acetyl

phosphate (Sigma Chemicals) and generating a standard curve from the resulting spectrophotometer readings at 540 num. Proteins assays were done on the cell-free extract using the BioRad protein assay kit (BioRad Laboratories, Richmond Calif.). Final activities were calculated on the basis of micromole of acetyl hydroxamate formed per minute per mg of protein. (Hydroxlyamine reagent is made immediately before the assay by adding 0.5 ml of 4M hydroxylamine hydrochloride to 0.5 ml of 4M potassium hydroxide. Ferric chloride reagent is: 10% ferric chloride, 3.3% trichloroacetic acid, 0.66N hydrochloric acid.)

Detailed Description Text - DETX (114):

Phosphotransacetylase levels were measured essentially according to the method described by Brown et al. in Journal of General Microbiology 102:327-336; 1977. Briefly, reaction constituents (Sigma Chemical Company) were prepared and added to a microcuvette as follows: 100 .mu.l of 1M Tris-HCl (pH8.0), 10 .mu.l of 500 mM magnesium chloride, 100 .mu.l of 5 mM NAD, 10 .mu.l of 50 mM CoA, 10 .mu.l of 500 mM L-malate, 10 .mu.l of malic dehydrogenase (Sigma #M-9004), 10 .mu.l of citrate synthase (Sigma #C-6987), 100 .mu.l of 100 mM acetyl phosphate, and 550 .mu.l of deionized water. The constituents were mixed, and placed in a Shimadzu UV-160 spectrophotometer (measuring at 340 nm) and zeroed against another cuvette containing the same constituents. The cuvette was removed, and 5 to 25 .mu.l of cell-free extract was added to the cuvette. The cuvette was quickly mixed, and returned to the spectrophotometer. The change in absorbance at 340 nm was measured for approximately 30 seconds in order to calculate the change in absorbance per minute. This number was then divided by the extinction coefficient of NADH, 6.22 liter/mmol.sup.-1 cm.sup.-1, in order to calculate the mmol of NADH formed per minute. Protein assays were also performed as described above and the final specific activity was determined in units of .mu.moles NADH formed per minute, per mg of protein.

Detailed Description Text - DETX (121):

The purpose of this experiment was to demonstrate that overproduction of the ack gene product could increase the percentage of 3-HV in the copolymer. Briefly, the ack gene was obtained from A. Nakata (Lee et al., Journal of Bacteriology 172:2245-2249; 1990) on plasmid pMKU814. This plasmid is a pUC derivative with a 1.4 kb fragment that contains the ack gene from the E. coli chromosome. A plasmid containing the PHB biosynthesis genes and the ack gene was constructed by removing the ack gene from pMKU on a 1.4 kb EcoR I-Pst I fragment, filling in the 5' overhangs utilizing the Klenow procedure (Maniatis, et al.) and ligating the filled-in fragment into pJM9131 that had been digested with Dra I (blunt-ended cut).

Other Reference Publication - OREF (1):

Peoples and Sinskey, "Genes to PHA Polymers," International Symposium on Biodegradable Polymers: p. 108, Oct. 29-31, 1991.

Other Reference Publication - OREF (28):

Peoples and Sinskey, "Fine structural analysis of the Zoogloea ramigera phbA-phbB locus encoding .beta.-ketothiolase and acetoacetyl-CoA reductase: nucleotide sequence of phbB," Molec. Microbio. 3: 349-357, 1989.

Other Reference Publication - OREF (30):

Peoples and Sinskey, "Poly-.beta.-hydroxybutyrate (PHB) Biosynthesis in Alcaligenes eutrophus H16: Indentification and Characterization of the PHB Polymerase Gene (phbC)," Journal of Biological Chemistry 264: 15298-15303, 1989.

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	11	glnap$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:38
L2	286	acetyl adj phosphate or acetylphosphate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L3	114394	promoter$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L4	1815924	induc$8 or regulat$8 or activat$8 or modulat$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L5	2	2 same 3 same 4	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L6	418982	acetate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37
L9	12633	isoprenoid$1 or lycopene$1 or carotene$1 or astaxanthin$1 or phytoene$1 or isopentyl adj diphosphate or ipp	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:43
L10	43684	polyhydroxybutyrate$1 or phb$1 or polyhydroxyvalerate$1 or phv$1 or polyhydroxyalkanoate$1 or pha$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L11	1246	polyketide$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L12	4	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 2	US-PGPUB; USPAT	OR	OFF	2005/06/15 15:20
L13	159	(9 or 10 or 11) near4 (gene$1 or sequence$1) and (6 same 4)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:09

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L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37
L9	12633	isoprenoid$1 or lycopene$1 or carotene$1 or astaxanthin$1 or phytoene$1 or isopentyl adj diphosphate or ipp	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:43
L10	43684	polyhydroxybutyrate$1 or phb$1 or polyhydroxyvalerate$1 or phv$1 or polyhydroxyalkanoate$1 or pha$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L11	1246	polyketide$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L12	4	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 2	US-PGPUB; USPAT	OR	OFF	2005/06/15 15:20
L13	159	(9 or 10 or 11) near4 (gene$1 or sequence$1) and (6 same 4)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:09
L14	45	(9 or 10 or 11) near4 (gene$1 or sequence$1) same (2 or 6)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:10

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	11	glnap$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:38
L2	286	acetyl adj phosphate or acetylphosphate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L3	114394	promoter$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:33
L4	1815924	induc$8 or regulat$8 or activat$8 or modulat$8	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L5	2	2 same 3 same 4	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L6	418982	acetate	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:34
L8	50	6 near8 4 near8 3	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:37
L9	12633	isoprenoid$1 or lycopene$1 or carotene$1 or astaxanthin$1 or phytoene$1 or isopentyl adj diphosphate or ipp	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:43
L10	43684	polyhydroxybutyrate$1 or phb$1 or polyhydroxyvalerate$1 or phv$1 or polyhydroxyalkanoate$1 or pha$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L11	1246	polyketide$1	US-PGPUB; USPAT	OR	OFF	2005/06/15 14:51
L12	4	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 2	US-PGPUB; USPAT	OR	OFF	2005/06/15 15:20
L13	159	(9 or 10 or 11) near4 (gene$1 or sequence$1) and (6 same 4)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:11
L14	45	(9 or 10 or 11) near4 (gene$1 or sequence$1) same (2 or 6)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:10
L15	931	(9 or 10 or 11) near4 (gene$1 or sequence$1) and 6	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:11
L16	51	15 and carbon adj (flux or flow)	US-PGPUB; USPAT	OR	OFF	2005/06/15 17:12